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During the second year of the funding period, we created human mammary epithelial cells (HMEC) with altered p53 or Rb activity by transducing human papillomavirus type 16 (HPV16) E6 or E7 gene, and examined them for known indicators of cellular immortalization and transformation, such as telomerase activation and response to TGF-β. In particular, we have demonstrated that early- and late-passages of HMEC contain different population of cell types showing remarkably different susceptibilities to E6 and E7, which target p53 and Rb functions, respectively. The difference is evident not only for immortalization of transduced cells but also for activation of telomerase during pre-crisis, that is prior to immortalization. This finding may provide clues to the roles of different oncogenes, tumor suppressor genes, and telomerase in the process of transformation of distinct types of breast cells. Experiments using pure populations of luminal and basal HMEC may provide direct insights into these mechanisms. We also describe the evidence that expression status of *bcl-2* may contribute to mammary tumorigenesis.

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(5) INTRODUCTION

Characterization of early events in the development of breast cancer is expected to bring a better understanding of how normal cells are transformed to malignancy and may suggest new strategies for detecting precancerous lesions and new treatment methods. Since germline mutations in two tumor suppressor genes, p53 and BRCA1, are associated with inherited predisposition to cancer (Malkin *et al.* 1990, Hall *et al.* 1990, Hollstein *et al.* 1991, Miki *et al.* 1994, Futreal *et al.* 1994), alterations in the p53 and BRCA1 genes represent some of the earliest genetic changes known to occur in the development of breast cancers.

To study the effects of inactivating mutations in the p53 and BRCA1 genes early in the breast-cancer pathway, we will develop genetically defined human mammary epithelial cell (HMEC) lines by introducing heterozygous and homozygous mutations of each gene by homologous recombination (Mansour *et al.* 1988, Capecchi *et al.* 1994). Additionally, we will construct HMEC lines deficient in p53 protein by expressing the E6 gene of human papillomavirus type 16 (HPV16), which increases the rate of degradation of p53 protein (Scheffner *et al.* 1990, Band *et al.* 1991). The consequences of these genetic changes for cell metabolism will be discovered through controlled *in vitro* comparisons between genetically altered derivatives and their isogenic parent cells.

(6) BODY

The following progress was made during the second year of the funded project (September 22, 1995 to September 21, 1996).

Collection of *in vitro* cultures of human mammary epithelial cells (HMEC) from women who are normal or who are known carriers of mutations in tumor suppressor genes.

We have established more than 90 primary HMEC cultures from breast tissue specimens, many of them from women who carry abnormal alleles of the BRCA1, BRCA2, APC, or NF1 genes (Appendices, Table 1). These cultures will become important and convenient resources for constructing homozygously mutant HMEC lines, since many of them already carry one mutated allele of a known tumor suppressor gene..

We have also established several primary ovarian cancer cultures from women who belong to BRCA1 families. Analysis of these cultures for the mutation status of BRCA1 alleles is in progress.

Construction of HMEC deficient in p53.

We originally proposed two approaches for constructing p53-deficient cells; i.e., by mutating the p53 gene directly or by abrogating the protein's normal cellular function. Only the second approach has been successful, and the reasoning is outlined below.

Approach #1: Construct HMEC containing homozygous deletions of p53 by homologous recombination.

The human *p53* gene, which is about 20 kb long, contains 11 exons and 10 introns (Appendices, Figure 1). Point mutations and small deletions have been found across the whole open reading frame, although hot spots cluster from exon 5 to exon 8. We have consrtucted knockout vectors (neoXBdt and neoPA(-)XBdt) which carry 12-kb homologous sequences that are disrupted by a neo gene and flanked by two copies of the *tk* gene (Appendices, Figure 1). A 4-kb DNA fragment covering a small portion of the huge intron 1, the whole exon 2 which contains the translation start codon, and part of intron 2 was deleted and replaced by a 2.67-kb complete neo gene cassette or a 1.25-kb neoPA(-) gene cassette.

Homologous recombination of the *p53* gene should yield a 10 kb (neoXBdt, neoXBP) or 8.6 kb (neoPA(-)XBdt) knockout allele in an XbaI digest, while the wildtype allele should give rise to a 10.6-kb band when a SmaI-XbaI DNA fragment from exon 11 is used as a probe. With an XbaI-EcoRI DNA fragment from exon 1 and intron 1 as the probe, a 7.5-kb knockout allele and a 11.2-kb wildtype allele should be detected in BamHI-digested genomic DNA from clones of heterozygous knockout cells.

Two transfection methods, calcium phosphate co-precipitation and lipofection, were tested in immortalized HMEC, 184A1 cells (Stampfer *et al.* 1985). Among several commercially available lipofection reagents, DOTAP (Boehringer Mannheim) showed best efficiency. Different ratios of DNA:DOTAP are known to affect transfection efficiency and should be optimized for each cell type. By fixing the amount of DOTAP (30μl) and changing the amount of DNA (linearized) from 1 to 10 μg, we found that toxicity to cells increased as the amount of DNA increased and that 5 μg DNA gave rise to more stable transformants (~15 colonies from 5×10^5 cells).

Positive selection with G418 and negative selection with ganciclovir are frequently used to select cell clones that have undergone homologous recombination. The optimal concentration of ganciclovir (2 μM) for negative *tk* gene selection in embryonic stem (ES) cells has been worked out by Mansour *et al.* (1988). To test the optimal ganciclovir concentration for negative selection in HMEC, we first introduced a neo*tk*/SK+ vector into the 184A1 cell line. Stable transformants were selected with 200 μg/ml G418. As shown in Appendices, Figure 2, 184A1 cells expressing the HSV-*tk* gene (neo*tk*/184A1) are more resistant to ganciclovir. Cell growth of neo*tk*/184A1 was not much affected at concentrations lower than 2 μM, and dropped dramatically only when up to 20 μM ganciclovir was used. At this concentration (20 μM), the growth of parental 184A1 cells was only slightly inhibited. Thereafter we used MCDB 170 or DFCI-1 complete medium containing 200 μg/ml G418 and 25 μM ganciclovir routinely for positive and negative selection in 184A1 cells. However, the enrichment factor of ganciclovir selection in 184A1 cells was only 1.5-2.0. A polyadenylation trap positive selection provided another 3- to 4-fold enrichment (neoPA(-)XBdt vs. NeoXBdt, data not shown).

Forty four clones from neoXBP transfection, 171 clones from neoXBdt transfection, and 120 clones from neoPA(-)XBdt transfection were obtained and subjected to Southern analysis. However, none of these 335 clones carried the knockout allele. Apparently, the frequency of homologous recombination is very low in 184A1 cells. One possible explanation for this low frequency of homologous recombination is the sequence mismatches between targeting vector and the recipient DNA due to polymorphisms. We are now consrtucting new targeting vectors using isogenic DNA fragments obtained from recipient cells.

Approach #2: Construct p53-deficient HMEC by introduction of human papillomavirus type 16 E6.

Interactions between the tumor supressor proteins p53 and Rb and transforming proteins of various DNA tumor viruses are thought to be essential components of the transformation process. The E6 and E7 proteins encoded by human papillomavirus type 16 (HPV16) bind and inactivate p53 and Rb proteins, respectively (Dyson *et al.* 1989, Scheffner *et al.* 1990, Werness *et al.* 1991). Binding of HPV16 E6 to p53 enhances degradation of the p53 protein, whereas binding of HPV16 E7 to Rb inactivates Rb by mimicking the hyperphosphorylated state, and thereby disrupting phosphorylated Rb interactions with other proteins. Thus, E6 and E7 effectively delete p53 and Rb without actually knocking out these genes. However, it should be noted that p53 and Rb are not only

proteins to bind to HPV16 E6 and E7. Carefully controlled experiments are necessary. Such experiments include analyses with an array of HPV16 E6 and E7 mutants that exhibit differential ability to abrogate p53 and Rb functions.

From Dr. D. A. Galloway, Fred Hutchinson Cancer Research Center, Seattle), we have obtained PA317 amphotropic packaging cell lines that express LXSN-based retroviral vectors (Miller *et al.* 1989, 1993) containing HPV16 E6 and/orE6 (Halbert *et al.* 1991). The viral supernatant from each packaging line, filtered through a 0.45-micron disposable syringe filter, was used for infections. Many of our HMEC cultures have been successfully transduced with E6 and/or E7 genes. We have confirmed the absence of p53 protein in E6-transduced HMEC by Western analysis (data not shown).

Construction of HMEC deficient in BRCA1.

Our original proposal to construct BRCA1-deficient HMEC by homologous recombination has been unsuccessful for the same reason described in p53 knockout experiments. As an alternative, we are attempting to down-regulate BRCA1 expression by addition of a BRCA1 antisense oligonucleotide (Thompson *et al.* 1995).

Characterization of HMEC transduced with HPV16 E6 and/or E7.

Telomerase activation.

Although common in rodent cells, spontaneous escape from senescence *in vitro* and the acquisition of an indefinite lifespan is an exceptionally rare event in human cells (Linder *et al.* 1990, Shay *et al.* 1989, 1991). The ability of the E6 and E7 genes of human papillomavirus type 16 (HPV16), which target p53 and Rb functions respectively, to immortalize human fibroblasts, keratinocytes, uroepithelial cells, and mammary epithelial cells has provided a useful model for studying the transformation process. However, as not all cell types are immortalized by HPV16 E6 and E7 with the same efficiency (Hawley-Nelson *et al.* 1989, Munger *et al.* 1989, Band *et al.* 1991, Halbert *et al.* 1991, Shay *et al.* 1991, Reznikoff *et al.* 1994), the growth control pathways targeted by HPV16 E6 and E7 appear to differ among cell types.

Human mammary epithelial cells (HMEC) derived from reduction mammoplasty tissues and grown in culture (Stampfer 1985, Band *et al.* 1989) are proliferative for several passages (designated as early-passage). This stage is followed by a period termed 'selection' when the majority of the cells become larger, flattened, and nonproliferative. If these cells are held in culture with continued feeding, a population of small, proliferative cells emerges and these cells remain proliferative for another 10-20 passages before senescence (designated as late-passage) (Stampfer 1985). In a recent detailed study, Wazer *et al.* (1995) showed that HMEC at different stages of culture had different susceptibilities for immortalization to HPV16 E6 and E7 oncogenes: expression of E6 resulted in efficient immortalization in late-passage HMEC, whereas expression of E7 immortalized early-passage HMEC.

While the mechanism that controls the timing of cellular senescence and immortalization remains unknown, increasing evidence (Morin 1989, Greider 1990, Hastie *et al.* 1990, Harley 1991, Blackburn 1991, 1992, Allsopp *et al.* 1992, Kipling *et al.* 1992, Levy *et al.* 1992) suggests that sequences at the ends of chromosomes (telomeres) act as molecular clocks. Telomeres, composed of TTAGGG repeats, become shorter as a function of aging *in vivo* (Allsopp *et al.* 1992, Hastie *et al.* 1990) and during the passage of normal cells *in vitro* (Counter *et al.* 1992, Harley *et al.* 1990). This loss of telomeric DNA is thought to contribute to senescence (Levy *et al.* 1992, Harley 1991). Activation of a mechanism to restore telomeres, namely telomerase activation, has been proposed as a critical event in the immortalization of human cells (Morin 1989, Greider 1990, Hastie *et al.* 1990,

Harley 1991, Blackburn 1991, 1992, Allsopp *et al.* 1992, Kipling *et al.* 1992, Levy *et al.* 1992). We chose to examine HMEC at different stages that were expressing HPV16 E6 and E7 together or separately from retroviral constructs (Halbert *et al.* 1991), with a view to establishing a temporal relationship between expression of different oncogenes, immortalization of different cell types, and activation of telomerase.

Different susceptibilities of early- and late- passage HMEC for immortalization to HPV16 E6 and E7 genes.

We introduced the HPV16 E6 and E7 genes, separately or in combination, into early-passage (initial outgrowth) and late-passage (post-selection) HMEC by retroviral infection, using the same viral titers and the same numbers of recipient cells (Appendices, Table 2). Following viral infection, cells were cultured in DFCI-1 medium containing 25 μg/ml G418 and examined for senescence, extension of life span, crisis, or immortalization (Appendices, Table 2). Despite the use of a different medium, our results were quite similar to those of Wazer *et al.* (1995) who used D2 medium which selects for outgrowth of immortal cells. E6 immortalized our late-passage HMEC very efficiently with a minimal (~1 week) crisis period, whereas E6-transduced early-passage HMEC gave rise to few immortal clones. In contrast, E7 was incapable of immortalizing late-passage HMEC, but E7-transduced early-passage HMEC resulted in a rapidly growing cell population, followed by a crisis period (4 weeks). Although many cells senesced during this crisis period, growing cells that emerged have been in culture for more than 70 passages. The combination of E6 and E7 led to a population of rapidly growing cells with a minimal (~1 week) crisis period, followed by efficient immortalization in both early-passage and late-passage HMEC.

Cell type-specific activation of telomerase by E6 and E7 in pre-crisis HMEC.

We examined telomerase activity in pre- and post-crisis HMEC using a modified version of the telomere-repeat amplification protocol (TRAP) assay. As shown in Fig. 1, those cells which became immortal (E7- and E6E7-transduced early-passage HMEC and E6- and E6E7-transduced late-passage HMEC) exhibited a high level of telomerase activity. Surprisingly, we have also detected telomerase activity at passage 4 after retrovirus infection when those cells were still in pre-crisis, that is, before immortalization. Cells transduced with vector control remained negative for telomerase activity. (Appendices, Fig. 3).

Serial dilution of cell extracts and a subsequent TRAP assay indicated that telomerase activity in pre-crisis cells was 5-15% of the level in each corresponding population of post-crisis immortal cells (Appendices, Fig. 4).

Telomerase is activated within one passage of retroviral infection.

To determine how soon telomerase is activated after introduction of HPV oncogenes, we performed TRAP assays of early-passage and late-passage HMEC within 1 passage of high-titer retrovirus infection. As shown in Appendices, Fig. 5, telomerase activity was detected in early-passage HMEC transduced with E7 alone, or with E6 and E7 together, as early as 2 days after infection. On the other hand, telomerase activity was detected within 2 days in late-passage HMEC transduced with E6 alone or with E6 and E7 together. Specificity of telomerase activation for cell type became more evident 8 days after infection. Transduction of the vector control did not activate telomerase in either cell type. However, it should be noted that in early-passage HMEC transduced with E6 alone, and in late-passage HMEC transduced with E7 alone, small but detectable amounts of telomerase activity were induced after high-titer infection of retroviruses.

These results suggest that cell type-specific telomerase activation occurred soon after the introduction of viral oncogenes, and was not due to a rare genetic event in a given cell population.

Clonal analysis of pre-crisis HMEC for telomerase activity, immortalization, and senescence.

To determine whether any differences in telomerase activity exist within a population of pre-crisis cells, we isolated 10 pre-crisis clones from E7-transduced early-passage HMEC. Six of them become immortal and four senesced. The TRAP assay for telomerase activity revealed that both immortalized and senesced clones expressed varying amounts of telomerase activity during their pre-crisis period (Appendices, Fig. 6). More importantly, some senesced clones expressed higher telomerase activity than some immortalized clones, suggesting that telomerase activation at pre-crisis might not be directly involved in the cellular immortalization process..

In contrast to our observation, Klingelhutz *et al.* (1996) recently reported that in early-passage HMEC, transduction of HPV16 E6 induced pre-crisis activation of telomerase but transduction of E7 had no effect. In our hands, although E6-transduced early-passage HMEC expressed detectable pre-crisis telomerase activity, the amount was significantly less than that expressed by E7-transduced pre-crisis early-passage HMEC (Appendices, Fig. 5). We do not have a good answer for these opposing observations; however, it is possible that the presentations of susceptible cell populations were different. This possibility may also explain another observation; i.e., telomerase activity in pre-crisis cells was approximately 5-15% of that in post-crisis cells in our experiments, as opposed to 1% reported by Klingelhutz *et al.* (1996).

Do our observations reflect the ability of E6 and E7 to target p53 and Rb functions? Klingelhutz *et al.* (1996) have shown that E6 also activates telomerase in pre-crisis keratinocytes, and that mutations in E6 that abrogate its ability to target p53 for degradation do not affect telomerase activation in keratinocytes. Recently, Dalal *et al.* (1996) analyzed a series of HPV16 E6 mutants for the ability to immortalize HMEC and to target p53 protein for degradation, and demonstrated that degradation of p53 is necessary for immortalization of HMEC. These results, although achieved using different cell types (HMEC vs. keratinocytes), suggest that E6-mediated telomerase activation at pre-crisis and subsequent cellular immortalization are caused by different underlying mechanisms; i.e., p53-independent and p53-dependent pathways. We do not know whether E7-mediated telomerase activation or immortalization of early-passage HMEC occur in an Rb-dependent manner. Experiments using a series of E7 mutants to map responsive domains for telomerase activation and immortalization are in progress.

TGF-β sensitivity.

Transforming growth factor β (TGF-β) inhibits growth of normal breast epithelial cells but dose not effect the proliferation of some of breast cancer cells (Hosobuchi *et al.* 1989). The loss of TGF-β response is thought to be an indicator of transformation and potentially could be a marker in the carcinogenecis pathway. Earlier studies with primary HMEC cell line (184) and immortalized HMEC cell line (184A1) showed full growth inhibition of 184 cells by TGF-β at 3.0 ng/ml, while 184A1 cells had varying levels of incomplete inhibition (10-30% of normal growth) (Hosobuchi *et al.* 1989). These results suggested that immortalized cells, such as those containing HPV16 E6 and/or E7, would have a different TGF-β growth response than the normal parental cells. Our preliminary results indicate that growth of late-passage HMEC immortalized with E6 and E6E7 is not inhibited by TGF-β , a characteristic not attributed to normal cells, suggesting that these cells may represent a stage along the carcinogenetic pathway. We are now performing a more detailed analysis of TGF-β sensitivity using various combinations of early- and late-passage HMEC and HPV16 E6 and E7 genes.

Overexpression of bcl-2 in HMEC.

Bcl-2, a protooncogene originally found in follicular lymphoma (Bakhshi *et al.* 1985), can prevent the programmed cell death (apoptosis) induced by withdrawal of growth factor in lymphocytes (Hockenbery *et al.* 1990, Deng *et al.* 1993) and neurons (Allsopp *et al.* 1993), or by serum deprivation in fibroblasts overexpressing *c-myc* (Bissonnette *et al.* 1992, Fanidi *et al.* 1992). This suggests that overexpression of *bcl-2* may be able to protect HMECs from programmed cell death and may contribute to the immortalization of HMECs.

Overexpression of bcl-2 in immortalized HMEC, 184A1.

We transfected 181A1 cells with bcl-2 overexpression vector, pCMVhubcl-2-neo (Appendices, Figure 7), and characterized three stable clones (4-1, 4-2 and 4-3). The *bcl-2* gene has three transcripts (8.5, 5.5 and 3.5 kb) in lymphocytes (Tsujimoto *et al.* 1986). No endogenous *bcl-2* mRNA was detected in 184A1 cells with a *bcl-2* DNA probe. A ~3 kb exogenous *bcl-2* mRNA was detected in clones 4-1 and 4-2, but not in 4-3 (Appendices, Figure 7). Consistent with results of Northern analysis, Western blots analysis showed that the 26-kd Bcl-2 protein was barely detectable in either the parental 184A1 cells or the 4-3 clone, and was expressed at high levels in the 4-1 and 4-2 clones (Appendices, Figure 7). Both 184A1 and clone 4-3 were used as negative controls for characterization of the *bcl-2*-expressing clones, 4-1 and 4-2.

Overexpression of *bcl-2* inhibits apoptosis in a variety of cell types, including lymphocytes, neurons, and fibroblasts (Hockenbery *et al.* 1990, Deng *et al.* 1993, Allsopp *et al.* 1993, Bissonnette *et al.* 1992, Fanidi *et al.* 1992). We first checked whether overexpression of *bcl-2* could protect 184A1 cells from 5-FUdR-induced apoptosis. As shown in Appendices, Figure 8, after 4-day treatment with 100 μM 5-FUdR ~50% of the 184A1 and 4-3 cells had died, while only ~20 % of 4-1 or 4-2 cells died of the treatment. Therefore, overexpression of *bcl-2* conferred 2.5-fold protection from 5-FUdR-induced cell death. Degradation of genomic DNA was taken as evidence of cell death by apoptosis. DNA fragmentation analysis was carried out in clones 4-2 and 4-3 (Appendices, Figure 9). Fragmented DNA was detectable by agarose gel electrophoresis analysis within the first 24 hours in 4-3, and increased within the following 24 hours of 5-FUdR treatment. In 4-2, no DNA fragmentation was observed within the first 24 hours of 5-FUdR treatment. It was detected within 48 hours, but was much less extensive than in 4-3. Thus DNA fragmentation was delayed in *bcl-2*-overexpressing clone (4-2) compared to the clone (4-3) which did not express exogenous *bcl-2*. Altogether, these data indicate that HMEC 184A1, like other cell types, are protected from induced apoptosis by overexpression of *bcl-2*.

$p21^{WAF-1}$ is a downstream mediator of p53-dependent apoptosis (el-Deiry *et al.* 1994). Recently, Upadhyay *et al.* (1995) reported that *bcl-2* inhibited p53-dependent apoptosis by suppressing the expression of $p21^{WAF-1}$, but did not affect the induction of p53 in response to DNA damage in MCF10A cells. By Western analysis (Appendices, Figure 10), we found that the level of $p21^{WAF-1}$ level was high in 184A1 cells, but barely detectable in *bcl-2*-overexpressing clones 4-1 and 4-2. Clone 4-3 showed intermediate levels of both Bcl-2 and $p21^{WAF-1}$. Therefore, in 184A1 cells, $p21^{WAF-1}$ is down-regulated by Bcl-2.

The extracellular matrix is important for organization and function of epithelial cells *in vivo*. Matrigel, a solubilized extracellular matrix extracted from Engelbreth-Holm-Swarm mouse sarcoma, consists of extracellular matrix proteins similar to those of the normal mammary basement membrane. Matrigel is often used for *in vitro* studies to mimic *in vivo* conditions. Bergstraesser *et al.* (1993, 1996) reported that normal and tumor-derived HMECs could be distinguished on the

basis of their morphology on Matrigel. When plated on Matrigel, primary normal HMECs form three-dimensional structures similar to the ductal and alveolar structures observed in the mammary gland, while malignant cells remain as single and move through Matrigel like tumor cells invading the basement membrane *in vivo*. Immortalized normal human mammary epithelial 184A1 cells also form duct- and alveolus-like structures on Matrigel. As shown in Appendices, Figure 11, our clones overexpressing *bcl-2* (4-1 and 4-2) did not form structures on Matrigel as efficient by as 184A1. Instead many cells remained separate, reminiscent of the behavior of the malignant cells described by Bergstraesser *et al.* (1993,1996). Clone 4-3 showed a phenotype similar to 184A1. Altogether, these data indicate that overexpression of *bcl-2* may not fully transform HMEC but does confer partially transformed phenotypes, and therefore contributes to mammary tumorigenesis.

Overexpression of bcl-2 in primary HMEC.

Due to the difficulty of transfecting primary HMEC, we looked for more efficient retroviral vectors to transfer genes into primary early-passage HMEC (BE46). We constructed two retroviral vectors, pLNPObcl-2 and pLXSNbcl-2, and introduced into amphotropic packaging cell line, PA317 (Miller *et al.* 1989, 1993). Immunofluorescence staining of PA317 stable transformants indicated that pLNPObcl-2 expressed Bcl-2 protein better than pLXSNbcl-2. Therefore, pLNPObcl-2 retrovirus was used for further studies. HMEC (BE46) at passage 1, grown in DFCI-1 medium, were infected with pLNPObcl-2 retrovirus and selected with 25-50 μg/ml G418. As illustrated in Appendices, Figure 12, infected cells proliferated well for about two weeks and then became enlarged and vacuolated and eventually senesced the same as the control cells. We therefore conclude that although overexpression of *bcl-2* does provide a survival advantage, it cannot by itself immortalize HMEC.

Immunofluorescence staining showed that Bcl-2 protein was barely detectable in parental BE46 cells, but was highly expressed in more than 90% of retrovirus-infected cells (pLNPObcl-2/BE46). Cytokeratin 19 (K19), an intermediate filament found in differentiated luminal epithelial cells, was highly expressed in most BE46 cells but was expressed at a lower level in fewer than 10% of pLNPObcl-2/BE46 cells. On the other hand, vimentin, an intermediate filament found in fibroblasts, was up-regulated and highly expressed in 50-70% of pLNPObcl-2/BE46 compared to 10-20% of BE46 cells (Appendices, Table 3). An epithelial-fibroblastoid cell conversion of this kind has been observed in several carcinoma cells both *in vivo* and *in vitro*, and is also found in mammary epithelial cells that are overexpressing oncogenes, such as *c-erbB2*. Lu *et al.* (1995) also observed the epithelial-fibroblastoid cell conversion when *bcl-2* was overexpressed in luminal epithelial cells immortalized by the SV40 large T antigen.

We are now testing whether *bcl-2* can cooperate with p53- or Rb-deficiency caused by expression of HPV16 E6 or E7, or with other oncogenes, such as *c-myc*, to change phenotypes in primary HMEC; i.e., immotalization, behavior in Matrigel, and expression of differentiation markers.

Isolation of pure populations of luminal and basal epithelial cells from human mammary gland.

Most breast cancers arise from epithelial cells of the mammary gland. Two types of epithelial cells are most common in the mammary gland: a continuous layer of glandular cells (luminal cells) lines the duct, whereas a discontinuous layer of cells near the basement membrane (basal cells) shares certain features of myoepithelial cells (Taylor-Papadimitriou *et al.* 1987). Intermediate phenotypes have also been observed. Basal cells predominantly express 5 types of keratins (K), K5, K6, K7, K14, and K17, whereas luminal cells predominantly express 3 types, K8,

K18, and K19 (Taylor-Papadimitriou *et al.* 1987, Trask *et al.* 1990, Bartek *et al.* 1991). Breast-cancer cells produce mainly K8, K18, and K19 (Trask *et al.* 1990). In order to characterize more details of breast cell types in regard to the experiments described above, we have established a system for isolating pure populations of luminal and basal epithelial cells from human mammary gland. Briefly, monoclonal antibodies specific to surface antigens of either luminal or basal cells were bound to magnetic microspheres coated with a second antibody. These specific antibody/bead complex were then used to directly isolate purified luminal or basal cells from human breast organoid cell preparations by magnetic separation (Gomm *et al.* 1995, O'Hare *et al.* 1991). We are now testing these pure populations of luminal and basal epithelial cells to establish temporal relationships among features such as expression of HPV16 E6 and E7, p53 and Rb functions, immortalization, TGF-β sensitivity, response to *bcl-2* overexpression, and activation of telomerase.

(7) CONCLUSIONS

Our work during the second year of the funding period has produced good progress on several aspects of the research outlined in our original proposal. HMEC with altered p53 and Rb activity were created by transducing HPV16 E6 and/or E7, and were examined for telomerase activation and response to TGF-β. In particular, we have demonstrated distinct pre-crisis telomerase activation in early- and late-passage HMEC by E7 or E6, which target Rb and p53 functions respectively, and we are preparing a manuscript. This finding may provide clues to the roles of different oncogenes (E6 and E7), tumor suppressor genes (p53 and Rb), telomerase activation, and immortalization in distinct types of breast cells. Experiments using pure populations of luminal and basal HMEC may provide direct insights into these mechanisms.

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(9) APPENDICES

Table. 1 Breast Tissue Samples.

BE-1	mastectomy
BE-2	BRCA-1 mastectomy
BE-3	mastectomy
BE-4	mastectomy
BE-5	mastectomy
BE-6	mastectomy
BE-7	mastectomy
BE-8	mastectomy
BE-9	mastectomy
BE-10	mastectomy
BE-11	mastectomy
BE-12	mastectomy
BE-13	mastectomy
BE-14	mastectomy
BE-15	punch biopsy
BE-16	mastectomy
BE-17	mastectomy
BE-18	mastectomy
BE-19	mastectomy
BE-20	NF-1 mastectomy
BE-21	APC biopsy
BE-22	mastectomy
BE-23	mastectomy
BE-24	mastecto;my
BE-25	NF-1 biopsy
BE-26	APC biopsy
BE-27	mastectomy
BE-28	mastectomy
BE-29	mastectomy
BE-30	mastectomy
BE-31	reduction mammoplasty
BE-32	reduction mammoplasty
BE-33	APC mastectomy
BE-34	APC mammotest biopsy
BE-35	fine-needle biopsy
BE-36	fine-needle biopsy
BE-37	fine-needle biopsy
BE-38	fine-needle biopsy
BE-39	fine-needle biopsy
BE-40	BRCA-1 mammotest biopsy
BE-41	mastectomy
BE-42	fine-needle biopsy
BE-43	fine-needle biopsy
BE-44	APC mammotest
BE-45	bilateral mastectomy
BE-46	reduction mammoplasty
BE-47	reduction mammoplasty
BE-48	mastectomy
BE-49	reduction mammoplasty
BE-50	reduction mammoplasty
BE-51	mastectomy
BE-52	mastectomy
BE-53	mastectomy
BE-54	reduction mammoplasty
BE-55	APC mammotest biopsy
BE-56	APC mammotest biopsy
BE-57	APC mammotest biopsy
BE-58	APC mammotest biopsy
BE-59	BRCA-1 mammotest biopsy
BE-60	prophylactic mastectomy
BE-61	mastectomy
BE-62	mastectomy
BE-63	mastectomy
BE-64	biopsy
BE-65	biopsy
BE-66	mastectomy
BE-67	mastectomy
BE-68	reduction mammoplasty
BE-69	mastectomy
BE-70	bilateral mastectomy
BE-71	mastectomy
BE-72	BRCA-1 mammotest
BE-73	mastectomy
BE-74	mastectomy
BE-75	BRCA-1 mammotest
BE-76	reduction mammoplasty
BE-77	BRCA-2 mastectomy
BE-78	mastectomy
BE-79	mastectomy
BE-80	APC mammotest biopsy
BE-81	mastectomy
BE-82	reduction mammoplasty
BE-83	mastectomy
BE-84	reduction mammoplasty
BE-85	reduction mammoplasty
BE-86	reduction mammoplasty
BE-87	mastectomy
BE-88	mastectomy - high risk
BE-89	mastectomy
BE-90	reduction mammoplasty
BE-91	biopsy - high risk
BE-92	mastectomy - high risk
BE-93	mastectomy
BE-94	bilateral mastectomy

Table 2. HMEC at early- and late-passages are immortalized with different efficiencies by E6 or E7.

HMEC	Retrovirus	Passages after infection at crisis, senescence or selection	Immortalization
Early-passage	LXSN	5	-
	LXSNE6	6	+/-
	LXSNE7	8	+
	LXSNE6E7	7	+
Late-passage	LXSN	12	-
	LXSNE6	14	+
	LXSNE7	13	-
	LXSNE6E7	15	+

Early-passage (initial outgrowth) and late-passage (post-selection) HMEC were infected with retroviral vectors, LXSN, LXSNE6, LXSNE7 or LXSNE6E7 and grown in DFCI-1 with 25μg/ml G418.

Table 3. Immunofluorescence staining of Bcl-2, cytokeratin 19 (K19), and vimentin in BE46 and pLNPObcl-2/BE46. '+' is an indicator if the intensity of staining. Numbers in parenthesis indicate the estimated percentages of positive staining cells.

	BE46	pLNPObcl-2/BE46
Bcl-2	±	+++ (>90%)
K19	+++ (90-100%)	++ (<10%)
Vimentin	++ (10-20%)	+++ (50-70%)

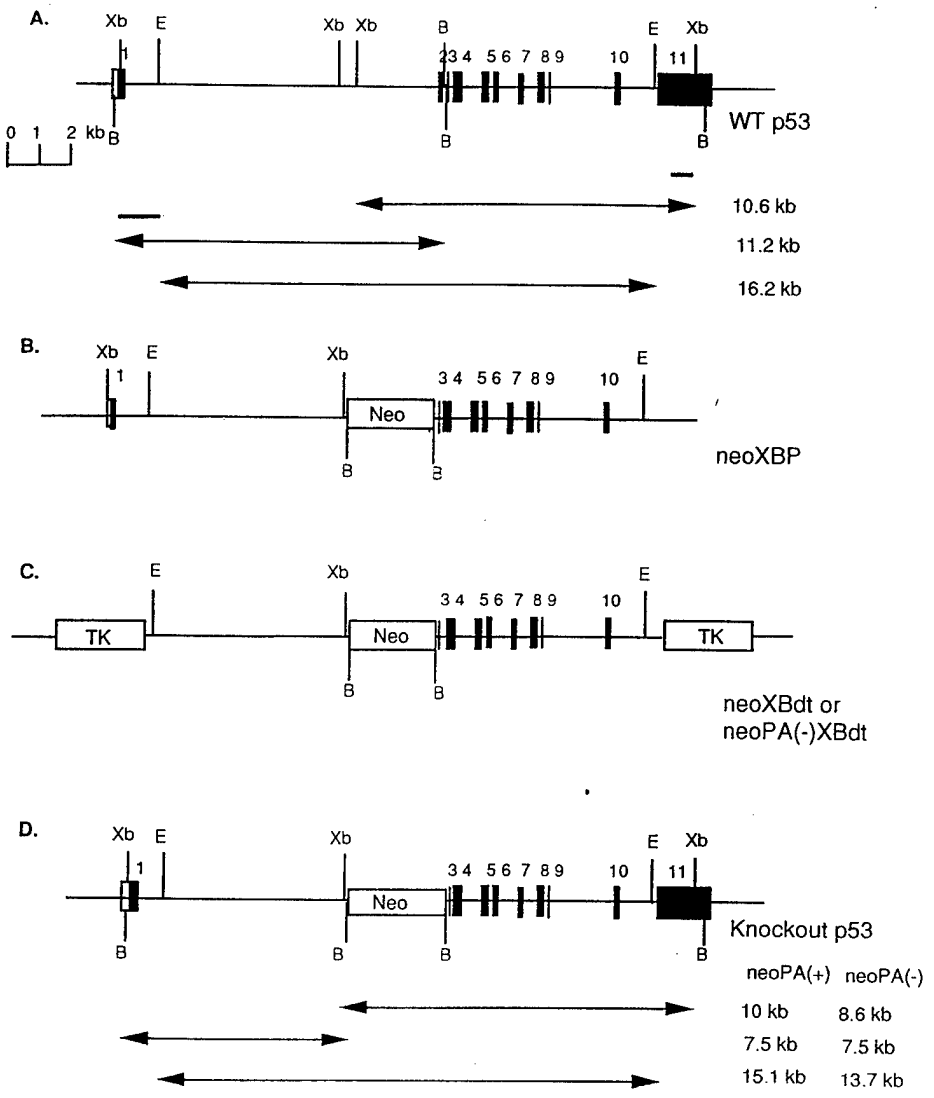

Figure 1. Genomic map of the *p53* gene (A), the *p53* knockout constructs: neoXBP (B), neoXBdt or neoPA(-)XBdt (C), and the *p53* knockout allele (D). Sizes of predicted DNA fragments from XbaI, BamHI or EcoRI digestion are indicated on the right. A SmaI-XbaI cDNA fragment from Exon 11 is used as a probe for XbaI digests. An XbaI-EcoRI DNA fragment from exon 1 and intron 1 is used as the probe for BamHI digests. Both sequences are flanking the sequences used for the construction of knockout vectors, therefore, can detect wildtype and knockout alleles only. *p53* cDNA probe is used to detect EcoRI digested DNA. B, BamHI; E, EcoRI; Xb, XbaI.

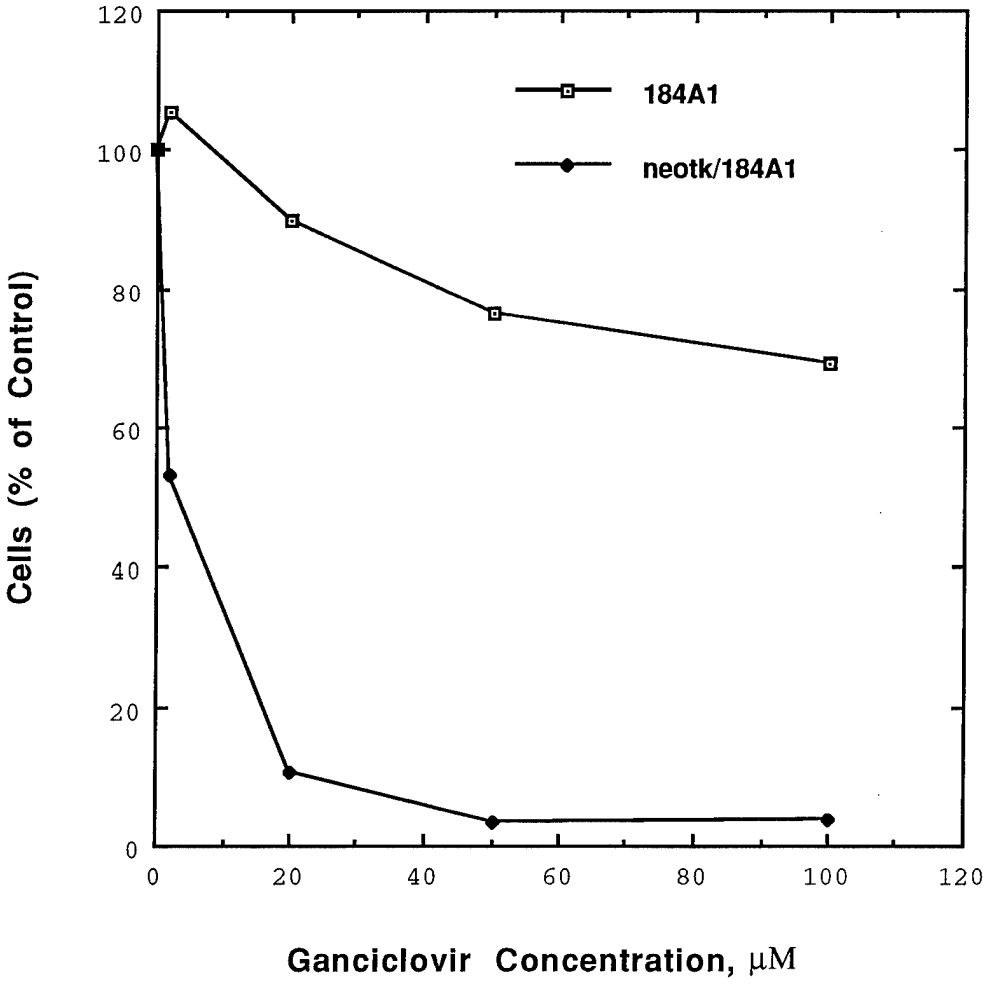

Figure 2. The effect of ganciclovir on cell growth of 184A1 and HSV-*tk*-transfected 184A1. Cell numbers in dishes with no ganciclovir were used as controls (100%).

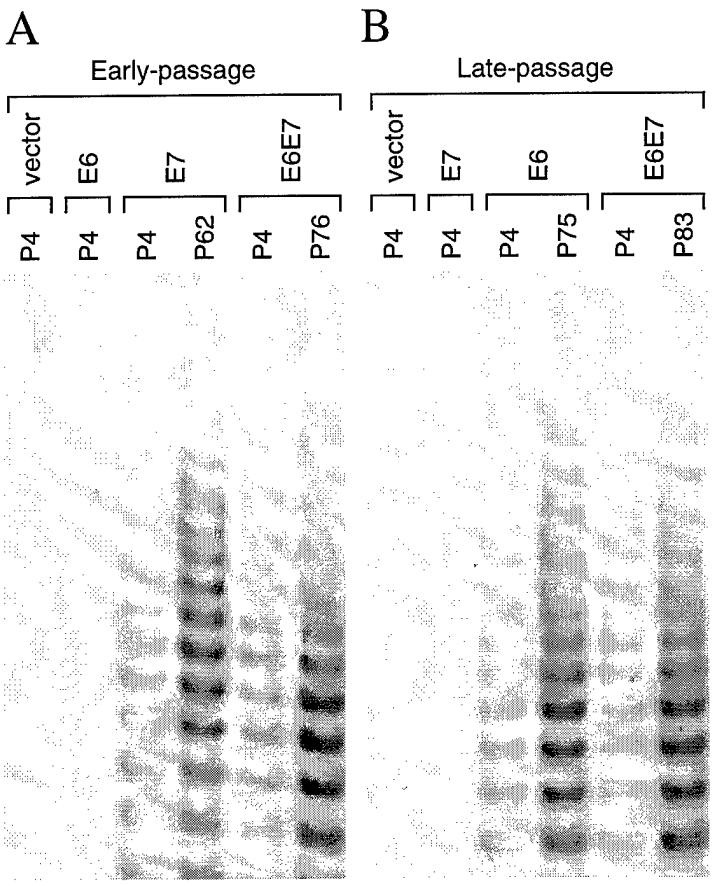

Figure 3. Telomerase activity in pre- and post-crisis HMEC transduced with HPV-16 oncogenes at early-passage (A) and late-passage (B) (see Table 1 for details). The cell extracts were prepared at indicated passage (P) number after each infection and then subjected to the modified version of the TRAP assay, using 5 μg of protein (Kim *et al.* 1994, Broccoli *et al.* 1995).

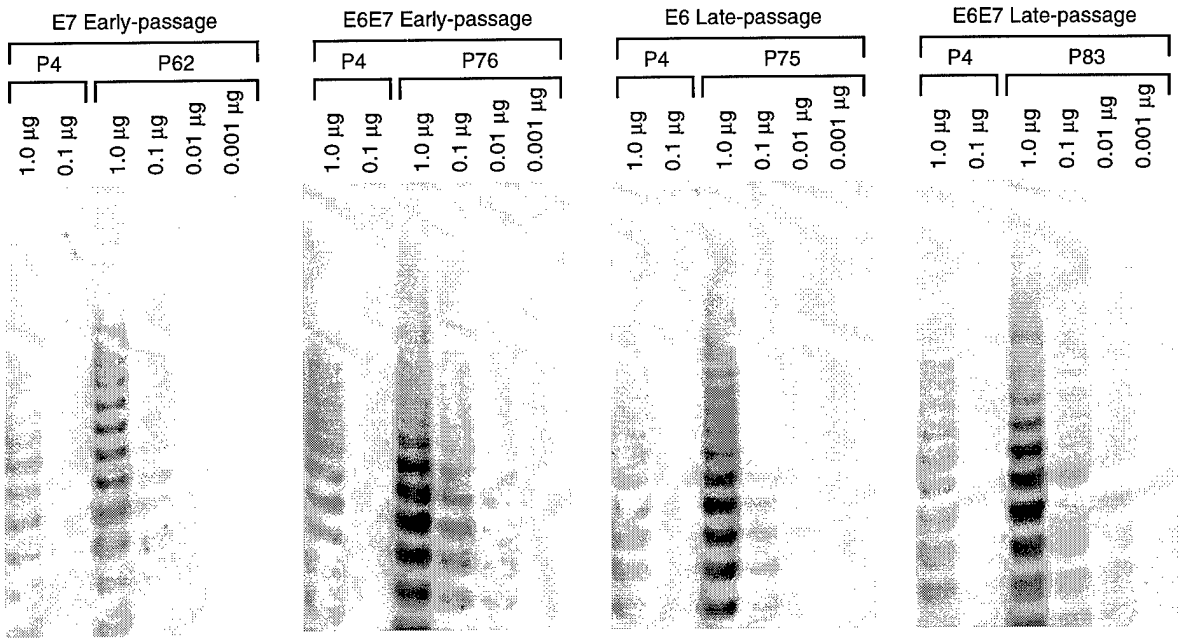

Figure 4. Dilution analysis of cell extracts to determine relative telomease activity. Extracts from telomerase-positive pre- and post-crisis cell populations documented in Fig.1 were diluted in lysis buffer (Kim *et al.* 1994) and the amounts shown were subjected to the TRAP assay. Quantification on a Phosphor imager showed that the assay was linear throughout the dilution and that telomerase activity in pre-crisis cells was approximately 5-15% of that in post-crisis cells (data not shown).

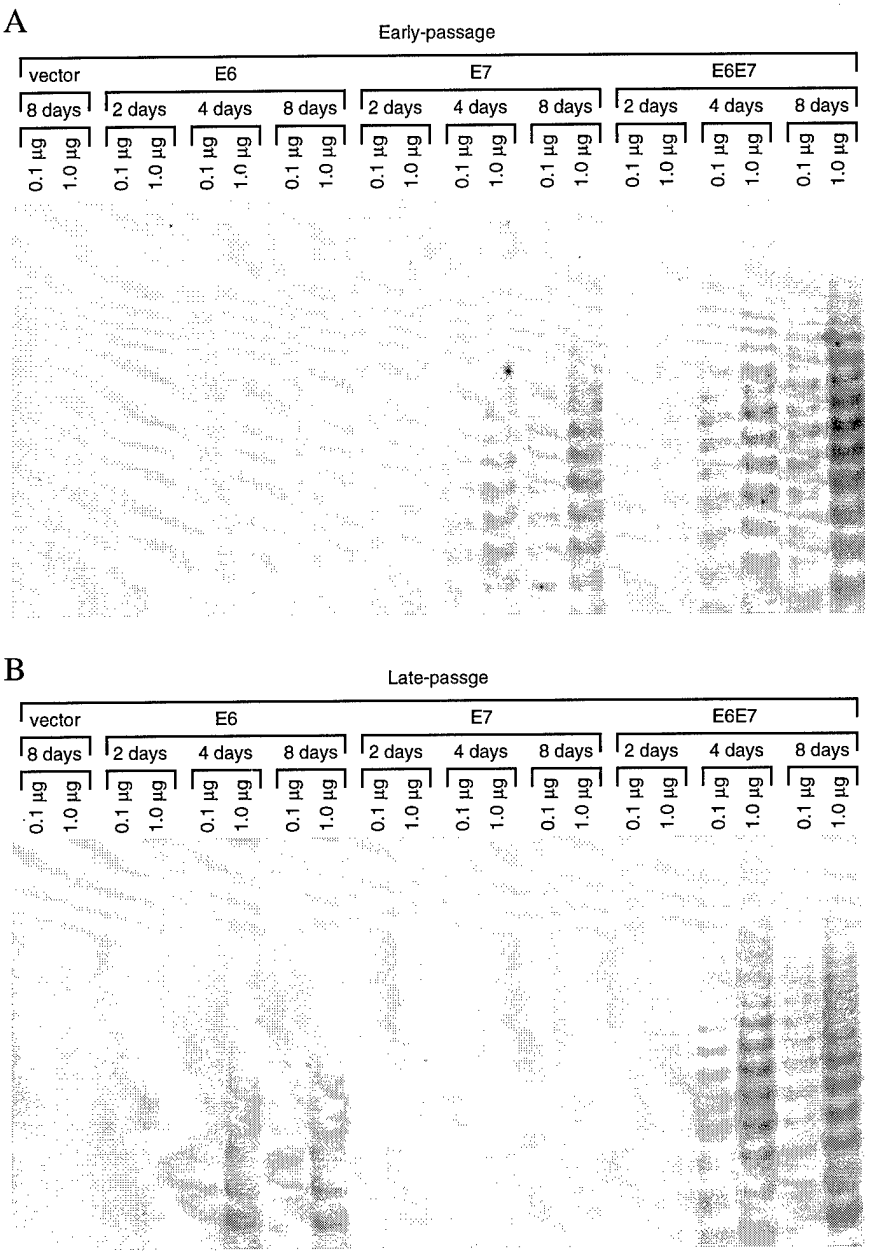

Figure 5. Distinct telomerase activation in early-passage and late-passage HMEC by E7 or E6. HMEC in early-passage (A) and late-passage (B) were plated in six-well polystyrene tissue culture dishes at approximately 10% confluency. Cells were incubated overnight with virus stocks of vector alone, E6, E7, or E6E7 construct at the m.o.i. (multiplicity of infection) of 20 so that most cells became infected. Cells were cultured without G418 selection and they became confluent by 8 days after infection. Cell extracts were prepared at 2, 4, and 8 days after infection and the amounts shown were subjected to the TRAP assay.

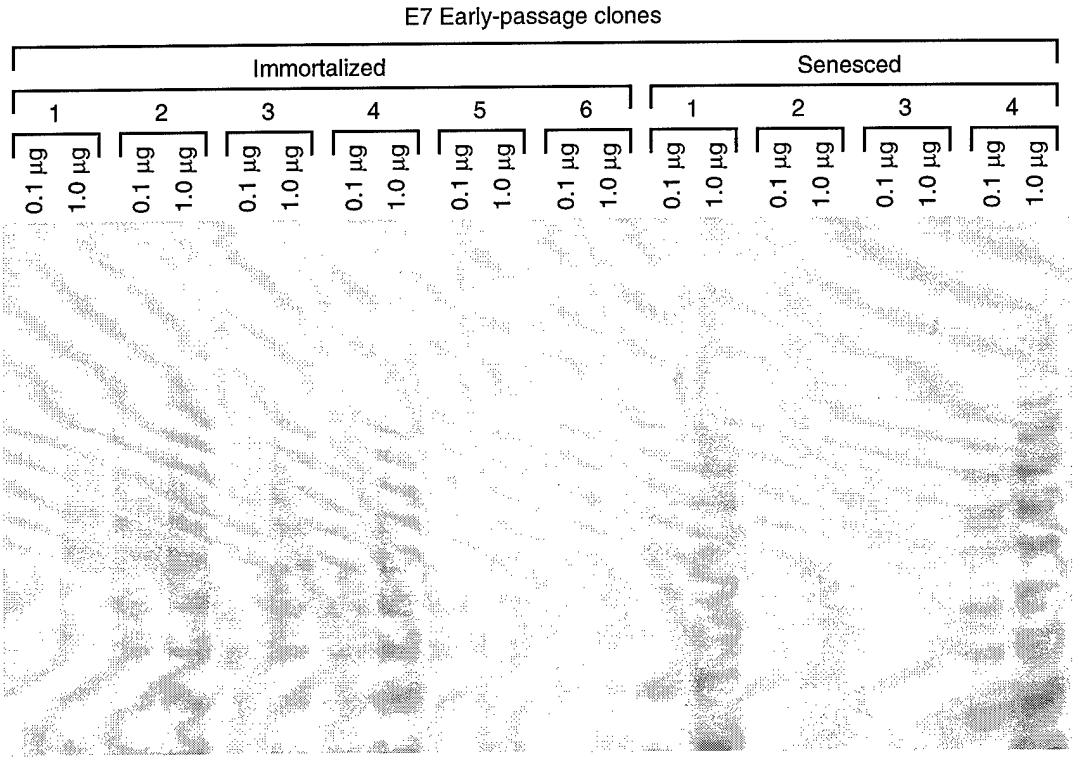

Figure 6. Clonal analysis of early-passage E7-transduced pre-crisis HMEC. G418 resistant colonies were isolated two weeks after transduction of E7 into early-passage cells. An aliquot of cells from each clone was used to prepare a cell extract at the next passage (4 weeks after infection), and the remaining cells were cultured further. Of 10 clones, four senesced during the next several passages and six became immortal. The corresponding cell extracts prepared earlier were divided into 'immortalized' and 'senesced' groups and were subjected to the TRAP assay.

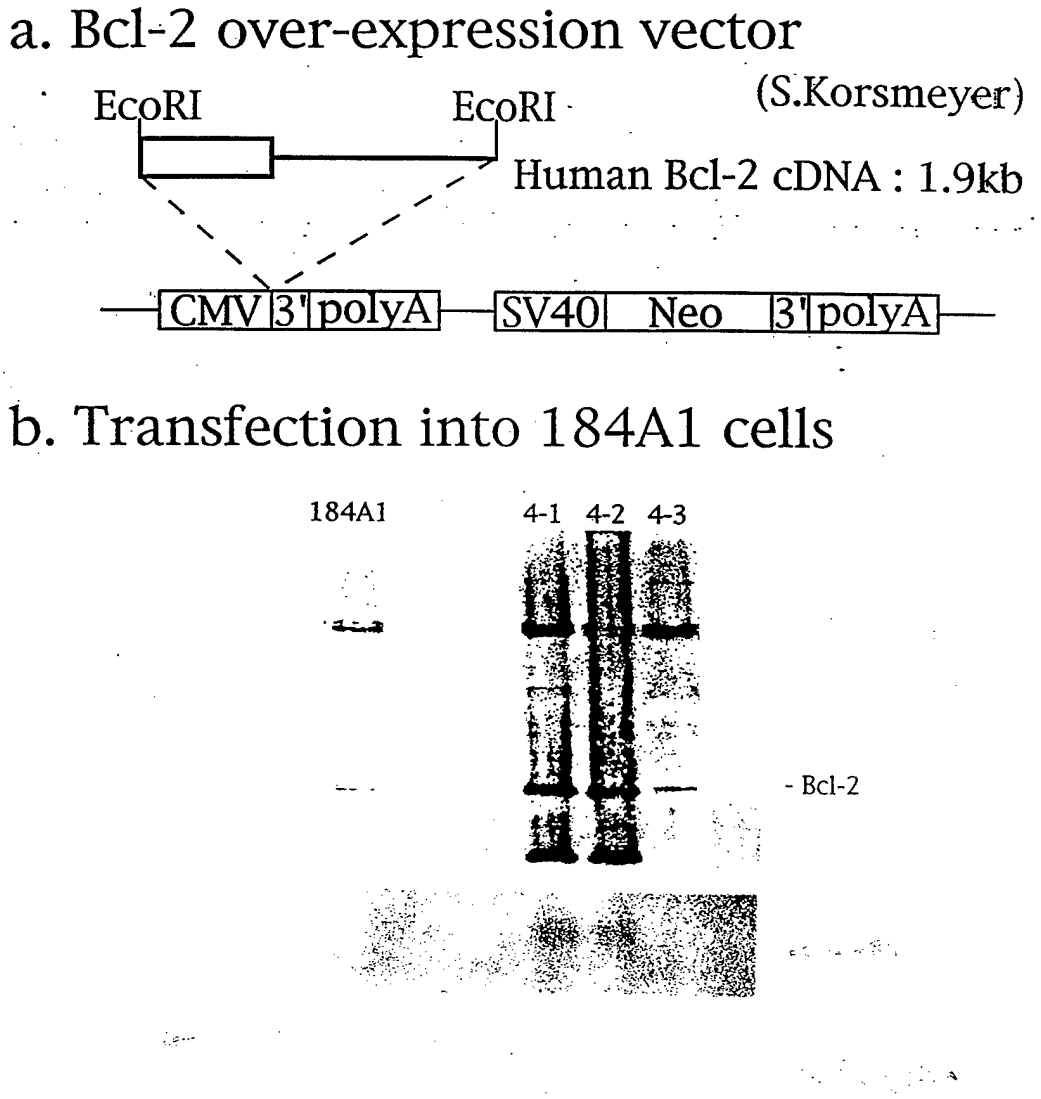

Figure 7. a) The bcl-2 expression vector, pCMVhubcl-2-neo; b) Western (upper panel) and Northern (lower panel) analyses of 184A1 and pCMVhubcl-2-neo-transfected 184A1 cell clones, 4-1, 4-2, and 4-3.

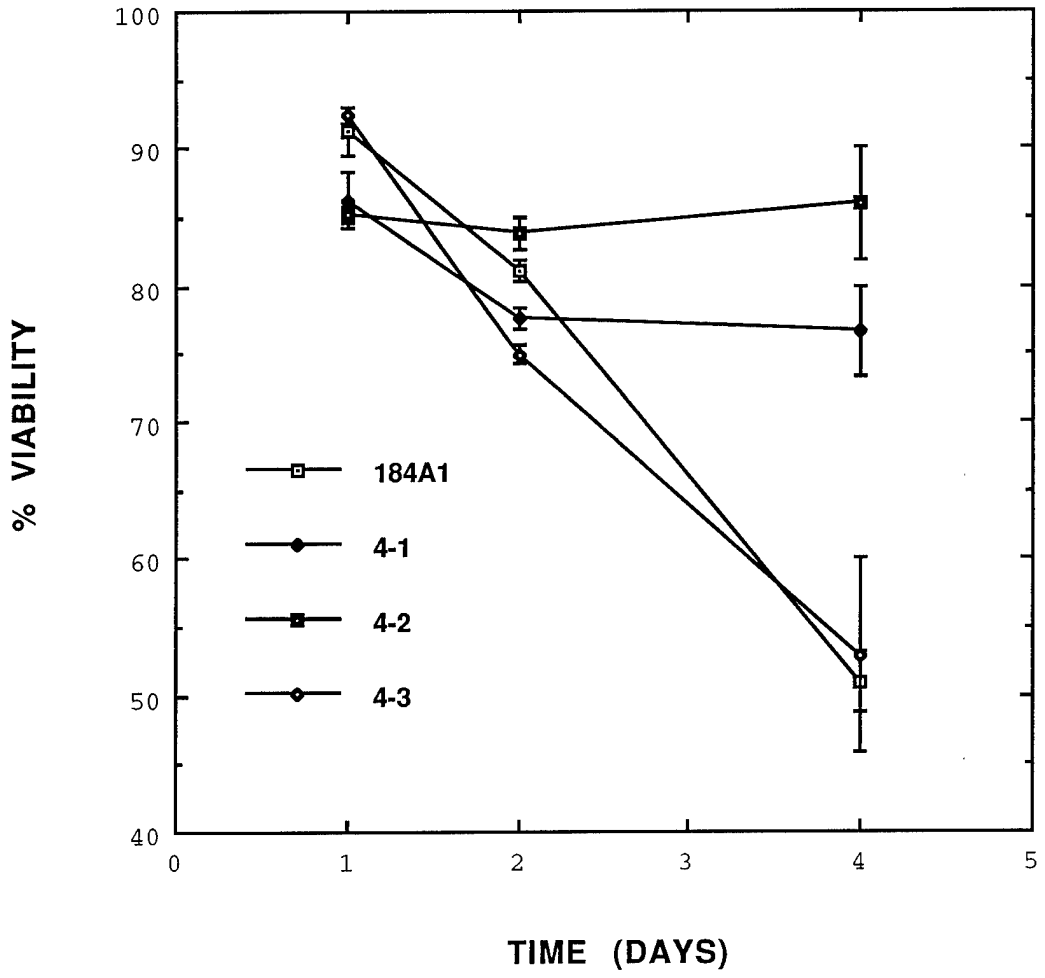

Figure 8. Cell viability of 184A1 and pCMVhubcl-2-neo-transfected 184A1 cell clones, 4-1, 4-2, and 4-3.

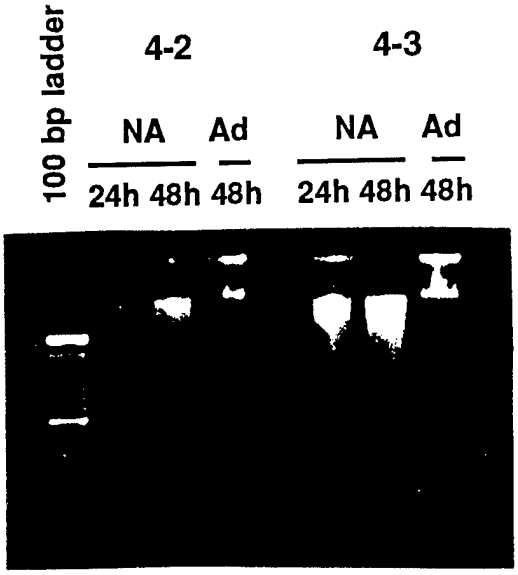

Figure 9. DNA fragmentation analysis of pCMVhubcl-2-neo-transfected 184A1 cell clones, 4-2 and 4-3. 'NA' indicates nonadherent, and 'Ad' indicates adherent cells.

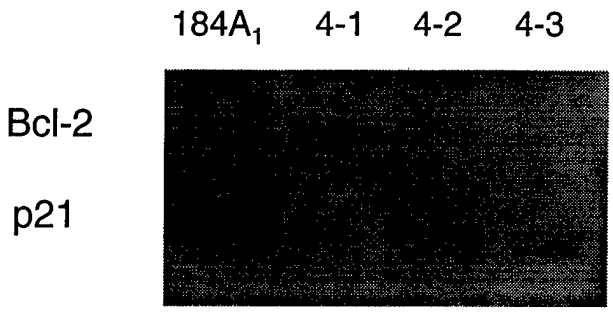

Figure 10. Western analysis of Bcl-2 and p21 *WAF-1*.

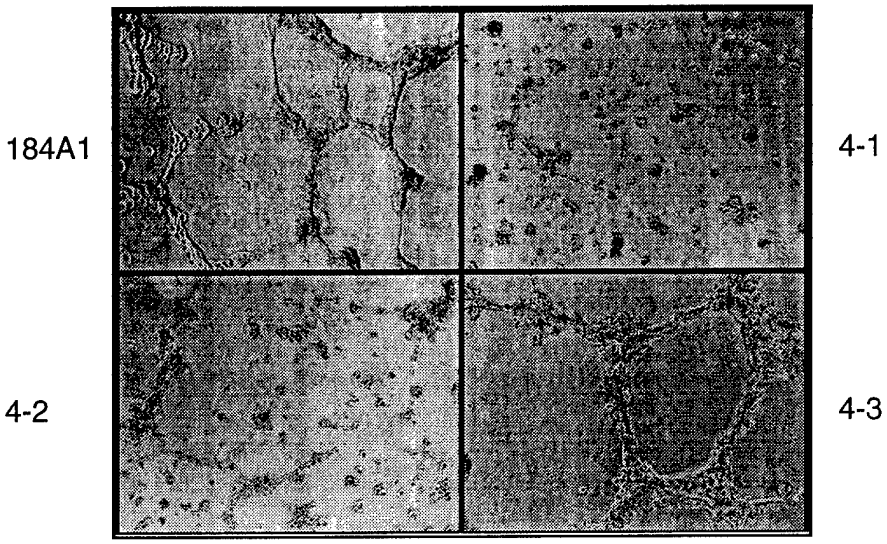

Figure 11. Structure formation of 184A1 cell clones on Matrigel.

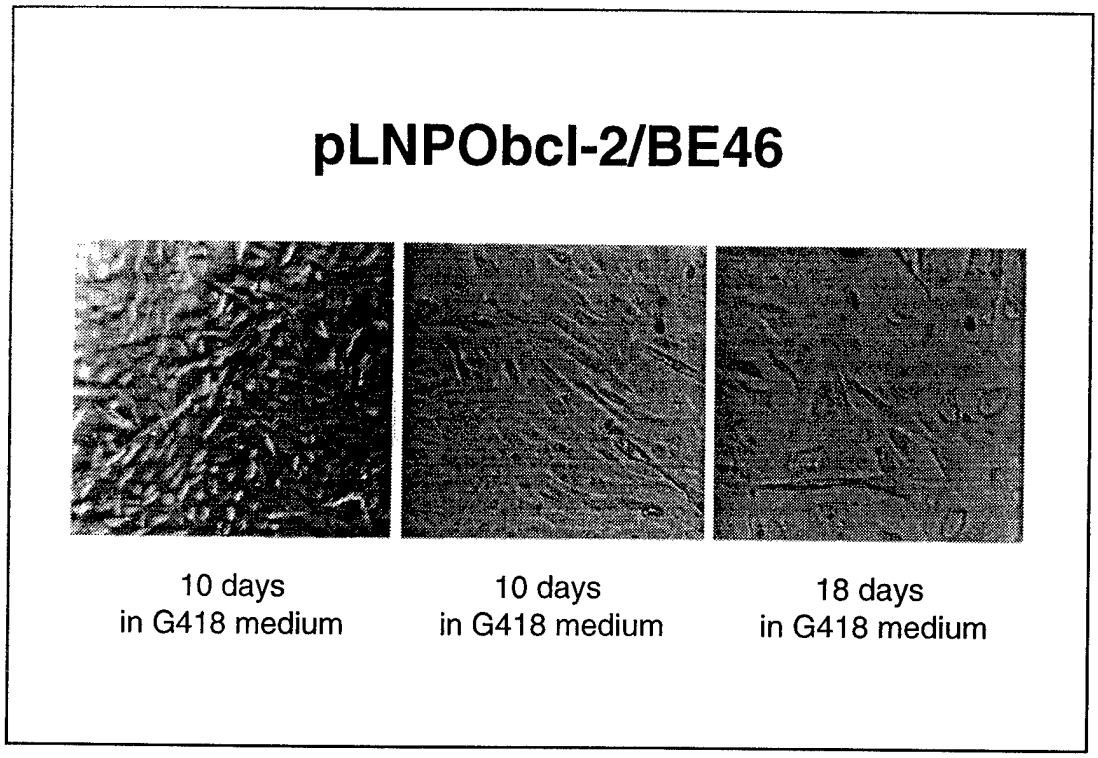

Figure 12. Morphology of pLNPObcl-2/BE46. Ten and 18 days in G418 selecting medium.

List of Salaried Personnel:

Name:	Position:
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